min. The pull through reaction is a PCR that uses primers which are at the extreme ends of the two DNA fragments being annealed in the assembly reaction. In this way, full length assembled product is amplified from the fragment mixture. An assembled product of the expected size (1.1 kb) was produced and gel purified. This product can be used directly as starting template for a coupled *in vitro* translation/transcription reaction.

Primers used (all written 5'-3'):

PEU (SEQ ID NO: 2)
AA TTC TAA TAC GAC TCA CTA TAG GGA GAG CAC TTC TGA TCC AGT CCG ACT GAG AAG GAA GGC CCA GCC GGC CAT GG

HA TAG (SEQ ID NO: 3)
TAC CCG TAT GAC GTG CCG GAT TAC GCA

T7 (SEQ ID NO: 4)
TAA TAC GAC TCA CTA TAG GGA GAG CAC TTC TG

HA mini (SEQ ID NO: 5)
TGC GTA ATC CGG CAC

Mycseq 10 (SEQ ID NO: 6)
CTC TTC TGA GAT GAG TTT TTG

Hismyc back (SEQ ID NO: 7)
GCA CAT CAT CAT CAC CAT CAC GGG GCC

c) Characterisation of the PCR assembled library on the basis of scFv expression

The scFv repertoire assembled with a glycine-serine tether was

A library of scFv fragments was generated by PCR amplification, as described (Example 3). Polyhistidine and myc tags were retained in the PCR fragments 3' to the scFv coding region. An origin of assembly-containing PCR fragment was generated by the ligation of two oligonucleotides as follows.

Oligonucleotides HA-OAS1 and HA-OAS2 were assembled together by the addition of 2 µl (approximately 100ng) of each oligo to 24µl 1 X TAQ buffer containing 1.5 µl of 5mM dNTPs and 0.5µl TAQ polymerase. The assembly reaction conditions were 94°C for 1 min, followed by 55°C for 4 min in 6 cycles. A pull-through reaction was set up consisting of 10µl of the assembly reaction added to 5 µl of scFv repertoire (approximately 500ng which had been PCR amplified with PEU and mycseq, Example 3), 5µl 5µM dNTPs, 5µl 10x PCR buffer, 2.5µl of HAmini primer (10µM), 2.5 µl PEU (10µM), and 0.5µl TAQ. PCR conditions were 25 cycles of 94°C 1 min, 55°C 1 min, 72°C 2 min. After pull-through reactions were complete a band of approximately 1.1 kb corresponding to assembled scFv and OAS tether was visible after gel electrophoresis.

HA-OAS 1 (135mer) (5'-3') (SEQ ID NO: 8):
TGC GTA ATC CGG CAC GTC ATA CGG GTA ACT ATT TTT CCC TTT GCG
GAC ATC ACT CTT TTT TCC GGT TCG AGA TCG AAA CTT TGC AAG CCT
GAT CGA CAT AGG GAC ATC TTC CAT GAA CTC ATC AAC GAC TTC TTC

HA-OAS 2 (no stop) (144mer) (5'-3') (SEQ ID NO: 9):
GAA CTC ATC AAC GAC TTC TTC TGT AAG TTC CAT GGG CCC TCC GTC
TCT CAC GTT TGT AAT CTT CTC TCT CAA ACC ATT CAG ATC CTC TTC
TGA GAT GAG TTT TTG TTC TGC GGC CCC GTG ATG GTG ATG ATG ATG
TCG GGC CGC

A version of primer OAS 2 was also produced which incorporated

a stop codon at the end of the myc tag. This oligonucleotide allows production of OAS-containing constructs which will not have the ability to form ARMs complexes because the presence of the stop codon will result in release of the ribosome.

HA-OAS 2 stop (5'-3') (SEQ ID NO: 10):

GAA CTC ATC AAC GAC TTC TTC TGT AAG TTC CAT GGG CCC TCC GTC TCT CAC GTT TGT AAT CTT CTC TCT CAA ACC **CTA** ATT CAG ATC CTC TTC TGA GAT GAG TTT TTG TTC TGC GGC CCC GTG ATG GTG ATG ATG ATG TCG GGC CGC

c) RNA transcription

RNA was generated by *in vitro* transcription of the PCR product. A transcription reaction was assembled by the addition of approximately 4µg of PCR product (in 20µl water) to 10 µl transcription buffer, 25mM rNTPs, and 5 µl Promega T7 enzyme mix. The reaction was incubated for 2 hours at 37°C. On completion of the reaction Dnase I was added and the reaction incubated for 15 min at 37°C. The transcription reaction was then phenol/choloroform extracted and divided into 4 aliquots of 12.5µl. 37.5µl of water was added to each aliquot and the RNA then ethanol precipitated by the addition of 5µl of 3M sodium acetate, 1µl glycogen and 125µl 100% ethanol. Precipitation was carried out at B70°C for 30 min, and the RNA then pelleted by centrifugation at 13 000 rpm for 10 min in a microfuge. Pellets were washed in 70% ethanol and resuspended in 50µl water. RNA was stored at B70°C.

d) Preparation of TMV Coat protein

The method of preparation of TMV coat protein was based on that described by Durham, 1972 J Mol Biol 67 289-305. The method involves dialysis of TMV in a high pH buffer (pH11) to disaggregate the coat protein from the viral RNA. This is

inventors have included the sections of MDV RNA in a ribosome display construct that generates RNA that can be replicated *in vitro*. Such a construct may also include the TMV or other viral OAS packaging sequence to allow encapsidation of the resultant RNA molecules. The design of a ribosome display construct incorporating MDV and OAS sequences is shown in Figure 6.

Primers to allow the incorporation of MDV RNA into the ribosome display construct are shown below:

The MVD1 replication site includes 63 nucleotides at the 5= end of the construct as follows (5'-3'): GGGGACCCCCCCGGAAGGG GGGGACGAGGTGCGGGCACCTCGTACGGGAGTTCGACCGTGACG (SEQ ID NO: 11).

This 63 nucleotide segment is then followed by the expression unit containing the scFv gene segments, detection and purification tags, the TMV OAS sequence if required and a tether. The 3′ end of the construct then includes the 3′ MDV sequence that is 156 nucleotides long as follows (5"-3'): CACGGGCTAGCGCTTTCGCGCTCTCCCAGGTGACGCCTCGTGAAGAGGCGCGACCTTCGTGC GTTTCGGTGACGCACGAGAACCGCCACGCTGCTTCGCAGCGTGGCTCCTTCGCGCAGCCCGC TGCGCGAGGTGACCCCCCGAAGGGGGGTTCCC (SEQ ID NO: 12).

The 3′ segment of the MDV sequence is too long to be made as a continuous oligonucleotide, so is split into two overlapping segments which can be made as single oligonulcoetides which can be annealed together. Three MDV oligonucleotides in total are required as follows:

MVD1 (encoding the 5′ 63 nucleotides of the MDV sequence followed by 23 nucleotides of the T7 promoter shown in bold)

(5'-3').

GGGGACCCCCCCCGGAAGGGGGGGACGAGGTGCGGGCACCTCGTACGGGAGTTCGACCGTGAC
G**AATTCTAATACGACTCACTATAG** (SEQ ID NO: 13)

MDV2: HA detection tag (bold face) followed by the first 79 nucleotides of the 3= segment of the MDV RNA.

Sense

**TACCCGTATGACGTGCCGGATTACGCA**CACGGGCTAGCGCTTTCGCGCTCTCCCAGGTGACG
CCTCGTGAAGAGGCGCGACCTTCGTGCGTTTCGGTGACGCACGA (SEQ ID NO: 14)

Reverse complement (5'-3')

TCGTGCGTCACCGAAACGCACGAAGGTCGCGCCTCTTCACGAGGCGTCACCTGGGAGAGCGC
GAAAGCGCTAGCCCGTG**TGCGTAATCCGGCACGTCATACGGGTA** (SEQ ID NO: 15)

MVD3: Remaining 77 nucleotides of the 3' MDV segment within an additional 19 nucleotide overlap (bold face) with MDV2 to allow assembly.

Sense

**GCGTTTCGGTGACGCACGA**GAACCGCCACGCTGCTTCGCAGCGTGGCTCCTTCGCGCAGCCC
GCTGCGCGAGGTGACCCCCCGAAGGGGGGTTCCC (SEQ ID NO: 16)

Reverse complement

GGGAACCCCCCTTCGGGGGGTCACCTCGCGCAGCGGGCTGCGCGAAGGAGCCACGCTGCGAA
GCAGCGTGGCGGTTC**TCGTGCGTCACCGAAACGC** (SEQ ID NO: 17)

<u>b) Assembly conditions</u>

VH CDR3 NMVRGVGRYYYMDV (SEQ ID NO: 18)
VL CDR3 CSRDSSGYHLV (SEQ ID NO: 19)

The off rate of this clone was measured by BiaCore and found to be $5 \times 10^{-3}$ $s^{-1}$.

*EXAMPLE 8*

*Use of the improved selection regime to selection for affinity matured variants of an antibody isolated against a GPI-linked cell surface receptor*

a) Mutagenised Libraries

A parental scFv that recognised the GPI-linked cell surface receptor of interest was isolated from a large phage display library using standard selection techniques. The parent clone had a $K_d$ of 0.02 $s^{-1}$, as measured by BiaCore analysis of FPLC purified monomeric scFv.

The VH CDR3 of the parent had the following sequence: VHNGWYALEY (SEQ ID NO: 20).
The VL CDR3 of the parent had the following sequence: NSWDSSGNHVV (SEQ ID NO: 21).

Libraries in which the central five residues of either the VH or VL CDR3 were mutated were generated by oligonucleotide mutagenesis and cloned into the ribosome display vector.

Libraries were designed as follows:

Library H4 (VH CDR3) VHNXXXXXEY (SEQ ID NO: 22)

Library L4 (VL CDR3) NSWXXXXXHVV (SEQ ID NO: 23)

Table 3

| Clone | Mutagenesised sequence (VH CDR3) | $K_d(s^{-1})$ | Fold improvement over parent |
|---|---|---|---|
| Parent | GWYAL (SEQ ID NO: 24) | 0.0203 | - |
| B1B3 | VNLLV (SEQ ID NO: 25) | 0.0233 | 0.87 |
| B1F12 | RSMDG (SEQ ID NO: 26) | 0.0283 | 0.71 |
| B2B4 | HAARR (SEQ ID NO: 27) | 0.0113 | 1.79 |
| B2H1 | RVRLL (SEQ ID NO: 28) | 5.9e-3 | 3.44 |
| B2B3 | FLSSI (SEQ ID NO: 29) | 0.0228 | 0.89 |

Table 4

| Clone | Mutagenesised sequence (VL CDR3) | $K_d(s^{-1})$ | Fold improvement over parent |
|---|---|---|---|
| Parent | DSSGN (SEQ ID NO: 30) | 0.0203 | - |
| C5 | SATHE (SEQ ID NO: 31) | 0.0166 | 1.2 |
| C10 | APHGS (SEQ ID NO: 32) | 0.0144 | 1.4 |
| A12 | TVNHD (SEQ ID NO: 33) | 0.0104 | 2.0 |
| D1 | HWQTD (SEQ ID NO: 34) | 7.4e-3 | 2.7 |
| H7 | NTSVT (SEQ ID NO: 35) | 2.5e-3 | 8.12 |